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(54) Abstract Title
Animal feed

(57) Animal feeds comprising a compound that disrupts the peptidoglycan layer of bacteria and a compound that disrupts the phospholipid layer of bacteria are disclosed. The feed is particularly active against Gram negative bacteria which possess both a lipopolysaccharide/lipid outer layer, and an inner peptidoglycan layer. The compounds can act synergistically against bacteria, and therefore improve the growth or the feed conversion ratio of animals. The feed may be fed to monogastric and/or non-ruminant animals such as poultry, pigs, piglets, (veal) calves or fish. The phospholipid disrupting compound can be a phospholipase, polyunsaturated fatty acid (PUFA, such as arachidonic acid) or a chelating agent (such as EDTA). The peptidoglycan disrupting layer can be an enzyme, such as an endoglycosidase or a peptidase, that can either hydrolyse peptidoglycan or cleave peptide crosslinks. The enzyme is preferably lysozyme.

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ANIMAL FEED

Field of the Invention

The present application relates to animal feeds, or additives or premixes therefor, that contain a compound that can disrupt the peptidoglycan layer of bacteria (e.g. lysozyme), and a compound that can disrupt the (inner and/or outer) phospholipid layer of bacteria (e.g. arachidonic acid, phospholipase). These two compounds can synergistically act as antimicrobial agents.

Background of the Invention

Farm, monogastric and/or non-ruminant animals such as pigs, piglets, poultry, calves, veal calves and fish are grown intensively for the production of meat, fish and eggs. These animals are fed diets containing a variety of raw materials of animal and/or vegetable origin to supply energy and protein. Most of the feed that is consumed is produced commercially by the compound feed industry, but a significant part is produced on the farm and fed directly. The feed is often supplemented with vitamins and minerals to meet the animal's nutrient requirements. The use of industrially produced enzymes in these feeds has now almost become common practice. Enzymes include phytases, amylases, proteases, glucanases, endoxylanases and mannanases. However, feed costs are the most important cost factor in animal production.

These enzymes are used to promote growth and feed conversion, and to reduce the environmental pollution produced by manure from pigs, poultry and fish. While antibiotics have been routinely added to animal feed, it has been reported that human pathogenic bacteria could develop a resistance against those or related antibiotics has been increasing rapidly. This has made it more difficult to cure people from bacterial infections, and the widespread use of antibiotics in animal feed has been blamed by various experts in the acceleration of build-up resistance to various antibiotics. This has led to a ban on the use of most antibiotics as growth promoters in animal feed in the European Union. It is likely

that other countries will follow these examples due to pressure from consumer and healthcare organisations. The feed industry is therefore more interested in natural additives with growth promoting effects, without any therapeutic side effects in humans.

WO-A-00/21381 (DSM N.V.) refers to animal feeds, which contain at least two antimicrobial enzymes and a polyunsaturated fatty acid (PUFA). One of these enzymes can be lysozyme. At that time it was not realised that a PUFA could disrupt the outer phospholipid layer of bacteria, and while it was known that the PUFA was beneficial, its mechanism was unknown, and therefore its use as a compound for disrupting the outer phospholipid layer was not disclosed in that document. Indeed this document does not contain a general disclosure of using a phospholipid-disrupting agent in animal feed.

Other documents that refer to antimicrobial effects for more than one component include:

(a) a combination of a chimeric peptides (a hybrid of cecropin and melittin) and lysozyme (Winans et al., Biochemistry 38: 11710 (1999)),

(b) a combination of lysozyme, hydrogen peroxide and ascorbic acid (T.E. Miller, J. Bacterial 98:949 (1969)), where the H_2O_2 oxidises the ascorbic acid;

(c) lysozyme and EDTA, hydrogen peroxide or ascorbic acid (Am. J. Physiol. Cell Physiol. 279:799 (2000)); and

(d) human secreted phospholipase A_2 and lysozyme or lysostaphin (Buckland et al., Biochimica et Biophysica Acta 1484:/95-26 (2000)), although animal feed is not disclosed and the combinations were not tested against live bacteria.

Description of the Invention

The present invention provides an animal feed, or an additive or premix composition therefor, comprising two components that show antimicrobial synergy. This may allow the improvement of growth and feed conversion ratio of farm, monogastric and/or non ruminant animals such as pigs, piglets, poultry, calves, veal calves and aquatic animals such as fish. This can allow one to reduce the amount of, or omit, an antibiotic (as a growth promoter).

The first aspect of the present invention relates to an animal feed composition, comprising:

(a) a compound that disrupts the peptidoglycan layer of bacteria; and

(b) a compound that disrupts the (inner and/or outer) phospholipid layer of bacteria.

Composition of the Bacterial Cell Wall

Based on colour formation following staining, bacteria can be divided into two classes, namely Gram positive and Gram negative bacteria. These two classes differ both in the composition and the structure of their cell walls. The major component for both Gram positive and Gram negative bacteria cell walls is peptidoglycan. The peptidoglycan is a thick rigid layer consisting of an overlapping lattice of two sugars that are cross-linked by amino acid bridges. The exact molecular structure of this layer is species specific.

The two sugars are N-acetyl glucosamine and N-acetylmuramic acid, which are linked through a ß-1,4-glycoside bond. Attached to N-acetylmuramic acid, which is a compound uniquely found in bacterial cell walls, is a side chain usually consisting of four amino acids. The most commonly found amino acids are L-alanine, D-alanine, D-glutamic acid, and a di-basic amino acid, usually diaminopimelic acid. The N-acetylglucosamine, N-acetylmuramic acid and the amino acid side chain forms a single peptidoglycan unit that can link with other units via covalent bonds to form a repeating polymer.

The polymer is further strengthened by crosslinks between the third amino acid (D-glutamic acid) of one unit and the fourth amino acid (diaminopimelic acid) of the next glycan tetrapeptide. The linker peptide of some bacteria contain glycine, serine and threonine. The degree of crosslinking determines the degree of rigidity. Peptidoglycan can be thought of as a strong woven mesh that holds the cell shape. It is not an impermeable barrier, since the openings in the mesh are large enough for many molecules to pass through them.

The cell walls of Gram positive bacteria consist almost entirely of the peptidoglycan layer, which forms a heavy cross-linked woven structure that wraps around the cell. It is very thick with peptidoglycan accounting for about 50% of the weight of the cell, and 90% of the weight of the cell wall. It is about 20 to 80nm thick.

The cell walls of Gram negative bacteria contain markedly less peptidoglycan, only 15 to 20% of the cell wall being made up of peptidoglycan, and this is only partially cross-linked.

Gram negative bacteria further differ from their Gram positive counterparts in that their cell wall contains an extra lipid layer. This is an outer membrane which primarily contains lipopolysaccharides (LPS), phospholipids, proteins and a small amount of peptidoglycan. It encloses the periplasmic space. The lipopolysaccharide consists of a core region to which are attached repeating units of polysaccharide. Components of this outer membrane are associated with endotoxic activity. In particular, the lipid portion of this membrane contains lipid A, a toxic compound that is responsible for most of the pathogenic effects associated with harmful Gram negative bacteria.

Compounds that Disrupt the Peptidoglycan Layer

This includes compounds that can lyse or degrade the cell wall. They may be able to cleave peptidoglycan. The compound may be a protein, such as an enzyme.

The compound may be an endoglycosidase, for example it may cleave or degrade peptidoglycan or glycopeptide. It may cleave ß-1,4-glycosidic bonds. In particular, it may cleave between the C1 of N-acetylmuramic acid and C4 of N-acetylglucosamine. Particularly preferred is lysozyme.

Other suitable enzymes are endoactylmuramidases (also fulfilled by lysozyme) and endoacetylglucosamidases, and exoacetylglucosamidases.

Preferably the compound is an enzyme, which is able to hydrolyse peptidoglycan from the bacterial cell wall. This may include glycosidases (for example which hydrolyse a polysaccharide chain), glucosamidases (for example endo-N-acetylglucosamidases), endo- or exo-peptidases (which can split peptide crosslinks) and peptidases or carbohydrases.

Enzymes that can therefore disrupt the crosslinking tetrapeptides, such as proteases, peptidases and amidases are included. These include acetyl muramyl-L-alanine amidases, intrapeptide hydrolases, lysostaphin (glycyl-glycine endopeptidase), L-alanine and glycine amino peptidase, L-alanine and glycine carboxypeptidase. Of these, N-acetylmuramyl-L-alanine amidase (NAMLAA, EC 3.5.1.28) is particularly preferred (this cleaves between polysaccharides and polypeptides). The human or mammalian form of this enzyme may be employed. Mucanolysin may also be used.

Carbohydrases include endoacetylmuramidases (which split the linkage N-acetylmuramyl-N-acetylglucosamine), endoacetylglucosaminidases (which split

N-acetylglucosaminyl-N-acetylmuramic acid) and exo-acetylglucosamidases. Acetyl-muramyl-L-alanine amidases can split the N-acetylmuramyl-L-alanine linkage. Peptidases include both endo-peptidases and exo-peptidases. These can split a variety of linkages, including alanyl-glycine, alanyl-alanine, alanyl-lysine, glycyl-glycine and alanyl-meso-DAP. The amount of the compound is preferably such that it is effective, e.g. antimicrobial, or that it can disrupt the peptidoglycan layer (of bacteria).

Preferably the enzyme is (e.g. freely) soluble in water. It may be thermostable.

Lysozyme is the preferred compound for disrupting the peptidoglycan layer. This may be provided in the animal feed at from 1,000 to 1,000,000 or 10,000,000, such as from 5,000 or 10,000 to 150,000 or 1,000,000, more preferably from 15,000 or 25,000 to 100,000 or 500,000 Shugar units per kilogram of animal feed. Preferably, this first enzyme may be present at an amount, by weight, to give a final concentration in the animal feed of from 0.04 to 44 milligrams per kg of feed, preferably from 0.2 or 0.4 to 6.7 or 20 milligrams per kg of feed, and more preferably from 0.8 or 1.1 to 4.4 or 10 (e.g. 1 to 5) milligrams per kg of feed, e.g. in the case of HEWL (Hen Egg White Lysozyme).

The lysozyme may be from a natural source, such as (e.g. hen) egg white. It may also be recombinant. For example, it may be expressed in an *Aspergillus* species, such as *Aspergillus niger*.

Other suitable compounds for disrupting the peptidoglycan layer include glycocalyx-disrupting compounds. This may be a bismuth salt.

Compounds that Disrupt the Phospholipid Layer

Such a compound may disrupt the inner and/or (preferably) the outer phospholipid layer. It may be a protein, such as an enzyme, or an organic or inorganic compound. The compound may be a surfactant or detergent, a chelating agent or an insect glycopeptide.

The amount of the compound may be an effective amount, e.g. antimicrobial, or that it can disrupt the phospholipid layer (of bacteria).

Suitable compounds include polyunsaturated fatty acids (PUFAs). A PUFA may be present in the animal feed at no more than 100 g, such as no more than 10 g, preferably no more than 1 g per kg of animal feed. Even lower concentrations of PUFA may be used, for example at least 0.0001g, such as at least 0.001g, preferably at least 0.002g per kg of feed. Suitable amounts are from 0.1 to 0.0001 g of PUFA per kg of feed, preferably from 0.02 or

0.05 to 0.002 g of PUFA per kg of feed and more preferably from 0.01 to 0.004 g of PUFA per kg of feed.

These amounts refer to the weight of the PUFA, and so if the PUFA is added in the form of an oil (e.g. having for example from 30 to 40% of the PUFA), then the amount of oil present (or added) can be calculated accordingly, for example by multiplying the amount of the PUFA by 100/X where X is the percentage of the PUFA in the oil. Hence, for example with a 30 or 35 to 40, 45 or 50% PUFA content, the amount of oil that can be added may vary proportionally, such as from 0.33 or 0.25 down to 0.00033 or 0.00025g of oil per kg of feed. Other amounts and intermediate ranges can be calculated on the same basis, starting with the figures for the PUFA in the previous paragraph.

The PUFA may be an Ω-3 or an Ω-6 PUFA. Preferably it is present in an oil, e.g. an edible oil. The oil may be microbial or single cell oil or a vegetable oil. Preferably the PUFA is in a liquid form, such as will occur if it is present in an oil. Preferred PUFAs are C_{18}, C_{20} or C_{22} (Ω-6 or Ω-3) PUFAs.

Preferred Ω3 and Ω6 PUFAs include:

(Ω3) docosahexaenoic acid (DHA, 22:6 Ω3), suitably from algae or fungi, such as the (dinoflagellate) *Crypthecodinium* or the fungus *Thraustochytrium*;

(Ω6) γ-linolenic acid (GLA, 18:3 Ω6);

(Ω3) α-linolenic acid (ALA, 18:3 Ω3);

conjugated linoleic acid (CLA, octadecadienoic acid);

(Ω6) dihomo-γ-linolenic acid (DGLA, 20:3 Ω6);

(Ω6) arachidonic acid (ARA, 20:4 Ω6); and

(Ω3) eicosapentaenoic acid (EPA, 20:5 Ω3).

Preferred PUFAs include ARA, DHA, EPA and/or GLA. ARA is particularly preferred. The PUFA may be from a natural (e.g. vegetable or marine source) or may be derived from a single cell, algal or microbial source.

A microbial oil may thus comprise an Ω3 or an Ω6 PUFA. The Ω3 PUFA (e.g. DHA)-containing oil may be a marine, e.g. fish (such as tuna) oil. The Ω6 and/or Ω3 PUFA (e.g. ARA, DHA or EPA)-containing oil can be a microbial or single cell oil.

An Ω6 and/or Ω3 PUFA-containing microbial oil (e.g. GLA, ARA and EPA) can be obtained from fungi, such as *Mortierella*, *Pythium* or *Entomophthora*. Ω3 PUFAs (e.g. EPA) can be produced from algae such as *Porphyridium* or *Nitzschia*. Preferably, the

microbial (or Ω6 or Ω3 (e.g. ARA, DHA or EPA containing)) oil can be produced by a single cell or a microorganism.This may be a bacteria, yeast, algae or fungi. Preferred fungi are of the order *Mucorales*. The fungus may be of the genus *Mortierella*, *Phycomyces*, *Blakeslea, Aspergillus, Thraustochytrium, Pythium or Entomophthora*. Preferred fungi are of the species *Mortierella alpina, Blakeslea trispora, Pythium insidiosum* and *Aspergillus terreus*.

Preferred yeasts are of the genus *Pichia* or *Saccharomyces*, for example *Pichia ciferrii*. Bacteria can be of the genus *Propionibacterium*. Preferred algae are dinoflagellate and/or belong to the genus *Crypthecodinium*, for example are of the species *Crypthecodinium cohnii*.

The Ω6 and/or Ω3 PUFA-containing oil may be a vegetable oil. These include blackcurrant, borage and primrose oils, and often contain an Ω6 PUFA, e.g. GLA. They also include olive, sunflower and soybean, soy (flower) oils, for example cooking and/or salad oils.

The PUFA may be in the free fatty acid form, or an ester form, such as a triglyceride. If it is present in an oil, then preferably at least 50%, such as at least 60%, or at least 70%, of the PUFA is in triglyceride form. However, the amount of triglyceride may be higher, such as at least 85%, 90% or at least 95% of the oil. Of these triglycerides, preferably at least 40%, 50% and suitably at least 60% of the PUFA is present at the α-position of the glycerol (present in the triglyceride backbone), also known as the 1 or 3 position.

The PUFA can be in the form of a free fatty acid, salt, fatty acid ester (e.g. a methyl or ethyl ester), as a phosopholipid and/or in the form of a triglyceride.

The PUFA may be present in or derived from a plant (or part of a plant, for example if the plant is transgenic or has been modified genetically).

The PUFA may act by being a surfactant. Other non-PUFA surfactants are contemplated, including tri-n-butylphosphate, cetylpyridinium chloride and/or glycerol mono-oleate.

In some embodiments it is preferred that the or each compound (e.g. PUFA) is still present inside the microorganism (that produced it). Hence the compound may be added as microorganism cells, such as biomass. The cells may be mixed with the animal feed (or with one or more feed substance(s) or ingredients). The microorganism may produce one

or more of the two types of compounds.

In a typical PUFA production (by fermentation) process the amount of PUFA produced may be from 7 to 10g/kg broth (i.e. wet biomass). Hence the amount of broth (we cells) to be added, or present in, the feed composition can be calculated by multiplying the amount of PUFA desired by a factor of 70 or 100 (e.g. 10g broth/kg feed gives a PUFA concentration of 0.1g/kg feed). If a dried biomass is added or used instead, then the dired cells can have a PUFA amount of 100 to 200, such as 140 to 180g/kg cells, and so to obtain the amount of PUFA one multiplies the amount of PUFA by 10 or 20 to give the amount of dried cells per kg feed.

The phospholipid-disrupting layer compound may be a peptide, such as a glycopeptide or a thiopeptide. The compound may thus be a cecropin, defencin, attacin, melittin, proline rich peptide, diptericin, pleurocidin, trichogin, alexomycin or nisin.

The compound may be a chelating agent, for example one bearing a negative charge. It may therefore chelate a positive ion, for example of valency II. This may include alkaline earth metals, such as calcium and magnesium. A suitable chelating agents include EDTA (ethylene diamine tetra acetic acid), CDTA, HDTA, NTA and/or IDA.

Other compounds include bile salts (such as deoxycholate, organic or inorganic acids (lactic acid, hydrochloric acid, ascorbic acid), other enzymes (rhodanese), imidazols (such as miconazol), or cinnamon aldehyde.

A further example of a phospholipid disrupting compound is the enzyme phospholipase A_2 (PLA_2). This may be from a mammalian, such as a human, source. The use of this enzyme in animal feeds is already known (EP-A-0,743,017). Other phospholipases may be used, and these include phospholipase A_1 (EC 3.1.1.32), phospholipase B (lysophospholipase), phospholipase C and phospholipase D. Phospholipase A_2 has the activity EC 3.1.1.4.

The phospholipase may be from a natural source, or it may be produced recombinantly, for example by expression of a heterologous gene in a microorganism, for example in *Kluveromyces lactis* or *Aspergillus*. The phospholipase may be present as an inactive pro-form that can be activated on ingestion, for example in the GI tract, suitably by proteolytic processing.

The phospholipase may be added to a feed at a concentration which varies according to the type of phospholipase employed, and the target animal. However, as a guideline, the

concentration of a phospholipase is from 1,000 to 5 million IU (International Units) per kg of phospholipid, such as from 10,000 to 500,000. The definition of the International Unit for phospholipase activity can be found in Example 1 of EP-A-0,743,017.

This enzyme may be present at a concentration to give from 5 to 5,000 , preferably from 10 to 2,500 , and more preferably from 50 to 1,000 Egg Yolk Units (EYU) per kilogram of animal feed. One EYU is defined as the amount of phospholipase enzyme that releases 1 μmol of acid per minute from egg lecithin at pH 8 and 40°C.

Thus, preferably the enzyme (or compound) may be present at an amount, by weight, to give a final concentration in the animal feed of from 0.005 to 5 (or 10 or 20) milligrams per kg of feed, preferably from 0.01 or 0.025 to 2.5 or 4 milligrams per kg of feed, and more preferably from 0.05 or 0.1 to 1.0 or 0.7 milligrams per kg of feed, for example in the case of pig pancreas PLA_2 produced in *A. niger*.

In the animal feed, the concentration of phospholipid may be from 0.5 g to 10.0 g per kg of feed. Consequently, the phospholipase may be present in a range of from 0.5 to 50,000 IU per kg feed, preferably from 5,0 to 5,000 IU per kg of feed. Of course, the dosage of phospholipase can be adjusted, if the phospholipid content of the feed is outside this range, or is not present at all.

Preferably the phospholipase is PLA_1. This has been expressed in various organisms, such as *E. coli*, *Saccharomyes cerevisiae* and *Aspergillus niger*. It is therefore expected successful (e.g. heterologous) expression of this phospholipase can be obtained in a wide range of microorganisms. PLA_2 hydrolyses phospholipids in the cell wall, such as phospholidylglycerol. Enzymes that have this activity (of PLA_2) can thus be used in the invention.

If the animal feed comprises a phospholipid, it is preferably a lecithin. If PLA_2 is used, then preferably then this is from a mammalian source, such as from a bovine, porcine, murine rat or human source.

Bacteria

As mentioned before, the animal feeds of the present invention are active against bacteria, in particular Gram negative bacteria, as these have both the lipid/lipopolysaccharide layer as well as the peptidoglycan layer. A non-exhaustive list of

Gram negative bacteria which the compositions in the present invention can be useful against is provided below.

Family (-)	Genera	Species
Acetobacteriaceae	Acetobacter, Gluconobacter, Frateuria	*A. aceti*
Alcaligenaceae	Alcaligenes, Deleya, Achromobacter	*A. faecalis*
Bacteroidaceae	Bacteroides, Porphyromonas, Fusobacterium, Leptotrichia and Selenomonas	*S. rumantium*
Chromatiaceae	Ameobobacter, Chromatium, Lamprobacter Lamprocystis, Thiocapsa, Thyocystis Thiodictyon, Thiopedia, Thiospirillum	*C. okenii*
Enterobacteriaceae	Escherichia, Salmonella, Shigella, Erwinia, Enterobacter, Serratia	*E. coli*
Legionellaceae	Legionella	*L. pneumophila*
Neisseriaceae	Neisseria, Kingella, Eikenella, Simonsiella Alysiella	*N. gonorrheae*
Nitrobacteriaceae	Nitrobacter, Nitrospena, Nitrococus, Nitrospira	*N. winogradskyi*
Pseudomonadaceae	Pseudomonas, Xanthomonas, Zoogloea, Fraturia	*P. aruginosa*
Rhizobiaceae	Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium	*R. laguminosarum*
Rickettsiaceae	Rickettsia, Rochalimae, Ehrlichia, Cowdria Neorickettsia	
Spirochaetaceae	Triponema, Borrelia	*T. pallidum*
Vibrionaceae	Vibrio, Aeromonas, Plesiomonas and Photobacterium	*V. cholerae*

Of these Gram negative bacteria, suitable are those of the genera Vibrio, Neisseria, or Salmonella.

Although effective against Gram negative bacteria, the compounds used in the invention can also be active against Gram positive bacteria. Suitable Gram positive bacteria are listed below.

Family(+)	Genera	Species
(Lacto)Bacillaceae	(Lacto)bacillus, Sporolactobacillus	*B. botulinum*
	Sporocarcina, Filibacter, Cayophanum	*B. cereus,*
	Clostridium, Desulphatomaculum	*B. coagulans*
		B. mycroides
		B. pumilis
		B. subtilis
		B. thuringiensis
Micrococcaceae	Arthrobacter, ~~and~~ Micrococcus	*M. luteus,*
		M. roseus,
		M. lysoeiktus,
		M. radiochrans
Mycobacteriaceae	Mycobacterium (including genus	
	Corynebacteria, Propionibacteria and	
	Bifidobacteria)	
	Nocardia, Rhodococcus	*M. tuberculosis*
Peptococcaceae	Peptococcus, Peptostreptococcus	*P. niger*
	Ruminococcuss, Sarcina, Coprococcus	

The Gram positive bacteria are suitably of the group *Cornynebacteria, Propionibacteria, Clostridia, Lactobacilli* and/or *Bifidobacteria.*

Animal Feed Compositions

The compounds, if enzymes, can be produced on industrial scale and/or may be recombinant. Lysozyme is commercially available, isolated from egg white, or may be recombinant. The enzyme may be naturally occurring or may be a (e.g. recombinant) variant or mutant thereof.

The compound is preferably recombinantly produced such as by expression of a heterologous gene or cDNA in a suitable organism, or alternatively by homologous (over)expression of a suitable endogenous gene. The glucose oxidase gene, for example, has been overexpressed in recombinant systems (WO-A- 89/12675, Chiron). Lysozyme (from egg white) can be recombinantly expressed by expression of the gene in *Aspergillus*

niger (Archer, D.B. *et al.*, Bio/Technology 8: 741-745 (1990)). A lysozyme mutant (produced by protein engineering) can also be used which may have better heat stability and/or stronger antimicrobial action.

A second aspect of the invention relates to a premix or additive composition to be added to one or more edible feed substance(s) or ingredient(s), for example to prepare or for supplementation feed composition (of the first aspect). This can comprise the two layer-disrupting compounds. Preferably the additive or premix comprises from 10 to 1000, such as from 25 or 50 to 750, preferably from 75 or 100 to 250 or 500, times as much of either of the two compounds as the feed. This is because the premix can be "diluted" by a factor of 10 to 1,000 (so that the premix constitutes 10% to 0.1% of final feed) when making the animal feed. This premix may be in the form of granules or pellets.

A third aspect of the invention relates to a process for the preparation of an animal feed composition, the process comprising adding to (or supplementing) an animal feed, or to one or more edible feed substance(s) or ingredient(s), the two layer-disrupting compounds.

The (or each) compound which disrupts peptidoglycan layer and/or phospholipid layer can be added to the animal feed composition separately from any feed substance(s) or ingredient(s), individually or in combination of other feed additives. Alternatively or in addition the or each compound can be in integral part of one of the food substances. The invention includes both preparing a feed composition with the two compounds or supplementing an existing feed composition with these two compounds.

A preferred method for the addition of the or each compound to the animal feed is to add the compound as transgenic plant material and/or (e.g. transgenic) seed. This is particularly suitable if the compound is protein, such as an enzyme. The compound may have been synthesised through heterologous gene expression, for example the gene encoding the desired enzyme may be cloned into a plant expression vector, under control of the appropriate plant expression signals, for example a tissue specific promoter, such as a seed specific promoter. The expression vector containing the gene including the enzyme can be subsequently transformed into plant cells. Transformed cells can be selected for regeneration into plants. The thus obtained transgenic plant can be grown and harvested.

Those parts of the plants containing the heterologous (plant) protein can be included in one of the compositions of the invention, either as such or after further processing.

Reference here is made to WO-A-91/14772, which discloses general methods for the (heterologous) expression of enzymes in (transgenic) plants. This includes methods for seed-specific expression of enzymes. The compound, such as the protein, may be contained in the seed of the transgenic plant. It may also however be contained in other plant parts such as roots, stems, leaves, wood, flowers, bark and/or fruit.

The addition of the compound in the form of a transgenic plant material, such as transgenic seed, may require the processing of the plant materials such as to make the compound available, or at least improve the compound's availability. Such processing techniques may include various mechanical techniques, such as milling and/or grinding, or thermomechanical treatments, such as extrusion or expansion. This may be conducted before any extraction of the compound, if necessary.

Exclusions

Preferably the animal feed of the invention does not contain any antibiotics. It may be free of a (supplementary or added) mineral component (such as zinc and/or iodine) and/or an immunomodulating agent (such as ascorbic acid). The composition may not include a combination of lysozyme, glucose oxidase and arachidonic acid or lysozyme, glucose, glucose oxidase and ascorbic acid. Other excluded compositions may include those comprising a combination of PLA_2 and lysostaphin, ascorbic acid and lysozyme, or EDTA and lysozyme.

Production of compounds by microorganisms

Although one or more of the compounds can be produced by a microorganism, for many situations (the producing) micro-organisms will not be added to or present in the feed, or at least live (or viable) organisms, such as bacteria, are not present in the feed. Hence in this case the composition is free from any microorganisms that produced one or more of these compounds (or micro-organisms from *Streptomyces*). Furthermore, the composition may be devoid of micro-organisms that produce lactic acid inside the animal (e.g. those of the genus *Lactobacillus* or *Enterococcus*). Typically, before addition of the compounds, the feed composition will be heated to kill, or reduce the number of, any bacteria present in the feed.

Uses of animal feed

A fourth aspect of the invention relates to a process for promoting growth, feed conversion or antibacterial activity, in a monogastric or non-ruminant animal, the process feeding the animal a compound that disrupts the peptidoglycan layer bacteria and a compound that disrupts the phosopholipid layer of bacteria. The animal can be fed the animal feed of the first aspect or feed preparable by the third aspect.

Suitable animals include farm, monogastric and/or non-ruminant animals such as pigs (or piglets), poultry (such as chickens, turkeys and laying hens), calves, veal calves or aquatic (e.g. marine) animals, for example fish.

A further aspect relates to the use of a composition of the second aspect as an additive for a monogastric or non-ruminant animal feed composition.

The compositions of the invention may be active *in vivo* (e.g. not *in vitro*), or only once ingested or inside the animal. The compounds may thus not be effective since the compositions may be too dry, e.g. they have a water content of no more than 10, 20 or 30 or 50%. Once ingested and inside the animal (e.g. in the stomach or rumen) there may be sufficient liquid (or water) for the compounds to become active or effective (e.g. antimicrobial, or layer disrupting).

Animal Feed Components

The compositions of the invention, in particular additive or premix compositions, can be either in liquid or solid form. If a solid, then this may be a powder, a granulate, extrudate or it may be pellets. For a solid form, the amount of water present may be below 20, 15 or even 10%, such as from 2 to 10%, 3 to 8% or 4 to 7%. The or each compound (e.g. enzyme) may be present at from 1 to 30%, such as 2 to 20%, for example 3 to 15%, and optimally at from 4 to 14% (on a dry matter basis). The remainder may comprise carbohydrates and/or carbohydrate polymers (such as starch and/or modified starch), for example at least 70, 80, 90 or 95%, such as from 75 to 90%. The composition may have a coating, for example if it is in a pellet, granulate, or extrudate form. There may thus be one or more coats on the outside of the composition, comprising one or more coating materials. If present, the coating (or coating materials) may be present at from 1 to 10%, such as from 2 to 6%, optimally at from 3 to 5%. The composition may have one or more stabilisers (such as glycerol and/or sorbitol) and/or one or more preservatives (such as sorbate and/or

benzoate).

If the composition is a liquid, then the water (or moisture) content will be higher. The water content may be up to 40, 50 or 60%, for example from 25 to 65%, optimally from 35 to 55%. If a stabiliser is present, this may be at an amount of from 45 to 65%, such as from 50 to 60%, optimally from 52 to 58%. The stabiliser is preferably sorbitol and/or glycerol.

A description of the preparation of pellets and granules, in particular carbohydrate based enzyme granulates, is described in WO-A-98/54980 (International Application No. PCT/EP98/03327), the contents of which is incorporated by reference.

The composition may comprise a carrier which may comprise at least 15% of an edible carbohydrate polymer. The carrier may be in particulate or powder form. However, if the composition is a liquid, it may be in the form of a solution or a slurry. The polymer preferably comprises glucose, or glucose-containing units, although it can contain glucopyranose units, amylose and/or amylopeptin. In addition, or instead of starch, a glucan, peptin or glycogen can be used. Preferably at least 15%, such as at least 30%, at least 40%, for example at least 60%, optimally at least 80% of the composition (or the solid carrier) comprises the carbohydrate polymer.

Additional details of enzyme-containing compositions for animal feed can be found in WO-A-98/55599 (International Application No. PCT/EP98/03328), the contents of which is also incorporated by reference. Although this document primarily deals with phytases, its teachings are equally applicable to other compounds, in particular enzymes.

Animal feed compositions of the first aspect will usually contain one or more feed ingredients or substances. These are ingredients and substances intended for consumption by an animal, and is therefore in a form suitable for ingestion and nutrition for an animal. This will therefore usually exclude human foodstuffs, or food substances or ingredients intended or destined for consumption by humans. Preferably the feed composition is both edible and digestible by the animal.

Suitably the substances and/or ingredients have a dry matter content of at least 80, 85, 90 or 95%. The protein content of the composition (or the substances and/or ingredients) may vary considerably, but may be from 5 to 20%, such as 10 to 15%, for example vegetable and/or plant products or parts thereof, such as buckwheat, rice, wheat, barley or corn. Substances or ingredients with higher protein contents, such as from 45 to

95%, e.g. 50 to 80%, may be provided, for example peanuts, poultry feathers, soy bean (or products thereof), sunflower (e.g. seeds) or casein. Preferred animal feed compositions may therefore comprise one or more of oats, pea (seeds), peanuts, soy beans, sunflower, canola, casein, coconut, corn, meat, millet, potato, rice, safflower and/or wheat. Preferably the composition (and substances or ingredients) have a crude fibre content below 30%, 25%, 20%, 15% or even below 10%. Similarly, the calcium content may be below 2%, such as 1%, below 0.5% and preferably less than 0.2%. The total phosphorous content of the (animal feed composition) is preferably from 2 to 0.01%, such as from 1 to 0.1%, optimally less than 0.5%.

The precise substances and ingredients can vary depending on the animal to be fed. An alternative composition may comprise one or more of bakery waste, sugar beet, brewers grain, canola, cassava, corn, fababean, fish (such as anchovy or herring meal), lentils, meat and/or millet.

Preferred features and characteristics of one aspect of the present invention are applicable to another aspect *mutatis mutandis*.

The present invention will now be described by way of example with reference to the following Examples, which are provided by way of illustration, and are not intended to limit its scope.

EXAMPLES

Characterization of antimicrobial compounds

Lysozyme obtained from chicken egg-white was obtained as a commercial product under the trade mark DELVOZYME™ from DSM Food Specialties, PO Box 1, 2600 MA DELFT, The Netherlands. The product contains 20 x 10^6 Shugar units/g product. One Shugar unit is defined as the amount of enzyme which causes a decrease of absorbance of 0.001 per minute at 450 nm and pH 6.2 at 25°C in a suspension of *Micrococcus lysodeikticus* (0.25 mg/ml) obtainable from Sigma Chemicals.

Phospholipase A_2 was obtained through production of pig pancrease PLA_2 in *Aspergillus niger* from DSM Food Specialties, Agri Ingredients, P.O. Box 1, 2600 MA Delft, The Netherlands. This process is described in WO-A-96/36244. Phospholipase concentrations are defined by Egg Yolk Units (EYU). One EYU is defined as the amount of phospholipase enzyme that releases 1 µmol of acid per minute from egg lecithin at pH 8

and 40°C.

Arachidonic acid (ARA) was obtained from DSM Food Specialties. Agri Ingredients, P.O. Box 1, 2600 MA Delft, The Netherlands under the trade mark VEVODAR™. This is a microbial oil (ARA content at least 35%) obtained by culturing the fungus *Mortierella alpina*.

Comparative Examples 1 to 3 and Example 4

Application of antimicrobial compounds in animal feed for poultry

Trials we carried out with broilers to test the efficacy of arachidonic acid (ARA) and lysozyme alone and the combination of both. Male and female broilers (Ross) were used in this trial; the animals were sexed and housed separated (4 pens per sex per treatment). Upon arrival, animals we weighed, and divided into floorpens equalising the average weights and its deviation between treatments. Fifteen animals were kept per pen. The pens were situated in an artificially heated, ventilated and illuminated broiler house. The floor space of each pen was 0.75 m^2; wood shavings were used as bedding material. The broiler house was illuminated for 23 hours per day. During the experimental period, light intensity was gradually reduced. The temperature was gradually reduced from 33°C the first day to 21°C at day 28. The animals had been vaccinated against New Castle disease and Infectious Bronchitis. The experiment lasted 28 days.

The experimental diets were offered *ad lib.* to the animals. Water was freely available. The feed was pelleted (with temperatures below 65°C) at a diameter of 2.5 millimeter.

The experiment comprised the following four treatments:

1) basal diet (negative control)
2) basal diet + lysozyme (50,000 Shugar units/kg of feed)
3) basal diet + arachidonic acid (ARA) to a final concentration of 0.001 g/kg of feed.
4) basal diet + lysozyme (50,000 Shugar units/kg of feed) + arachidonic acid (ARA) to a final concentration of 0.001 g/kg of feed.

Gain and feed conversion were measured. The composition of the feed (basal diets) used was:

Ingredients	Content (%)

Rye	10
Wheat	41.85
Soy oil	2
blended animal fat	6
Rape seed meal	7.5
Soya bean meal (45.4% crude protein)	18.5
Full fat toasted soya beans	5
Soya isolate	2.5
Corn gluten meal	2.5
Vitamins/premix	1
Limestone	1.4
Monocalciumphosphate	1.2
Salt (NaCl)	0.25
L-lysine.HCL	0.14
DL-methionine	0.16
ME broilers (MJ/kg)	12.02
Crude protein (%)	22.4
Crude fat (%)	10.3
Lysine (digestible, %)	1.06
Methionine + cystine (digestible, %)	0.78

The lysozyme and arachidonic acid were added to this basal diet by mixing them first with a carrier.

The effects of the lysozyme and arachidonic acid on growth and feed conversion ratio in broilers after 28 days are shown below in Table 1.

Table 1

Example	Diet	Feed Intake (g)	Growth (g)	Feed conversion ratio
1	Basal diet	2173	1319	1.65
2	Basal diet + lysozyme	2169	1322	1.64
3	Basal diet + arachidonic acid	2261	1413	1.60
4	Basal diet + lysozyme + arachidonic acid	2249	1409	1.59

A synergistic effect was found for the combination of ARA and lysozyme on the feed conversion ratio.

Comparative Examples 5 to 7 and Example 8

Application of antimicrobial compounds in animal feed for piglets

Crossbred piglets (equal number of barrows and gilts; in total 80 animals) of a similar age and weight were used in this trial. They were housed in environmentally controlled rooms, and had *ad lib.* access to feed and water at all times. Temperature, ventilation and illumination were applied according to common practice. The piglets were allotted to one of four treatments. There were two piglets in each pen with 10 replications (weight blocks) per treatment. The sexes were divided evenly over the treatments. The trial started 2 weeks after weaning of the piglets, at an age of approximately 38 days, and lasted for 6 weeks.

Body weight and pen feed consumption were measured after three and after six weeks of the experiment.

The basal diet was a typical corn-soybean meal diet, with the following composition:

Raw Material	Content (%)
Corn	63.4
Soyabean meal	33.73
Dicalcium phosphate	1.29
Limestone	0.74

Salt (NaCl)	0.33
L-lysine.HCl	0.01
Vitamin-trace mineral premix	0.5

The diet was calculated to contain 3265 kcal ME/kg, 20.5% crude protein, 1.15% lysine and 0.68% methionine + cystine. No antibiotic was added to the feed.

The experiment comprised the following treatments (Examples 5 to 8):

5) basal diet (negative control);

6) basal diet + lysozyme (100,000 Shugar units/kg of feed);

7) basal diet + arachidonic acid to a final concentration of 0.001 g/kg of feed.

8) basal diet + lysozyme (100,000 Shugar units/kg of feed) + arachidonic acid to a final concentration of 0.001 g/kg of feed.

The results obtained in terms of feed intake, growth and feed conversion ratio are shown below in Table 2.

Table 2

Average effects of lysozyme and ARA on growth and feed conversion ratio in piglets (38 to 80 days of age).

Example	Diet	Daily Feed Intake(g)	Daily gain (g)	Feed Conversion Ratio
5	Basal diet	621	371	1.69
6	Basal diet + lysozyme	622	375	1.66
7	Basal diet + arachidonic acid	662	395	1.67
8	Basal diet + lysozyme + arachidonic acid	649	403	1.61

The combination of two different types of antimicrobial compounds (i.e. arachidonic acid and lysozyme) resulted in a surprising synergistic effect on feed conversion ratio.

Comparative Examples 9 to 11 and Example 12

The use of the antimicrobial compounds lysozyme and arachidonic acid in fish nutrition.

Effects of supplemental antimicrobial compounds on growth and feed conversion ratio were studied with trout (*Oncorhynchus mykiss*).

The diet composition used was as follows:

Raw material	Content (%)
Soyabean meal	41
Soya beans, pressure cooked	20
Wheat gluten	22
Fish oil	13
L-lysine-HCl	0.8
DL-methionine	0.2
Vitamin/mineral premix	3.5

No growth promoting antibiotic was added to the feed.

Experiments were conducted with 200 trout with a mean initial body weight of 9.1 g/trout which were allotted to 4 equal groups. Diets were fed to these 4 groups over a period of 49 days. The water temperature was kept constant at 15°C. The diets were fed twice daily to satiation avoiding feed losses. Weight gain and feed conversion ratio were determined.

The experiment comprised the following treatments (Examples 9 to 12):

9) basal diet (negative control);

10) basal diet + lysozyme (50,000 Shugar units/kg of feed);

11) basal diet + arachidonic acid to a final concentration of 0.001 g/kg of feed.

12) basal diet + lysozyme (50,000 Shugar units/kg of feed) + arachidonic acid to a final concentration of 0.001 g/kg of feed.

The results obtained, in terms of growth and feed conversion, are shown below in Table 3.

Table 3

Gain, feed intake and feed conversion ratio in trout fed for 49 days on diets supplemented with lysozyme +/- arachidonic acid.

Example	Diet	Feed Intake (g/trout)	Gain (g/trout)	Feed Conversion Ratio
9	Basal diet	16.4	11.2	1.46
10	Basal diet + lysozyme	16.3	11.3	1.44
11	Basal diet + arachidonic acid	18.1	12.6	1.44
12	Basal diet + lysozyme + arachidonic acid	17.8	12.8	1.39

The results obtained demonstrate the favourable effects of a peptidoglycan disrupting compound and phospholipid disrupting compound on growth and feed conversion ratio in trout.

Comparative Examples 13 to 15 and Example 16

The use of antimicrobial compounds Lysozyme and Phospholipase in fish nutrition

Effects of supplemental antibacterial enzymes on growth and feed conversion ratio were studied with trout (*Oncorhynchus mykiss*). Set-up, diet composition, housing, etc. were similar to those described in Examples 9 to 12.

Experiments were conducted with 200 trout with a mean initial body weight of 9.1 g/trout which were allotted to 4 equal groups. Diets were fed to these 4 groups over a period of 49 days.

The experiment comprised the following treatments (Examples 13 to 16):

13) basal diet (negative control)

14) basal diet + lysozyme (50,000 Shugar U/kg of feed)

15) basal diet + phospholipase A_2 (PLA_2; 500 EYU/kg of feed)

16) basal diet + lysozyme (50,000 Shugar U/kg of feed) + phospholipase A_2 (500 EYU/kg of feed).

The results obtained, in terms of growth and feed conversion, are shown below in Table 4.

Table 4

Gain, feed intake and feed conversion ratio in trout fed for 49 days on diets supplemented with lysozyme and/or phospholipase A_2.

Example	Diet	Feed Intake (g/trout)	Gain (g/trout)	Feed Conversion Ratio
13	Basal diet	16.4	11.2	1.46
14	Basal diet + lysozyme	16.3	11.3	1.44
15	Basal diet + phospholipase	16.0	11.3	1.42
16	Basal diet + lysozyme + phospholipase	17.1	12.3	1.39

The results obtained demonstrate again the synergistic results of a peptidoglycan disrupting compound and phospholipid disrupting compound on growth and feed conversion ratio in trout.

Example 17: *in vitro* tests

In vitro tests were carried out to substantiate the synergistic antimicrobial effect of a combination of a peptidoglycan layer disrupting compound and a (inner and/or outer) phospholipid layer disrupting compound.

In disk diffusion (also called Bauer-Kirby) susceptibility tests, small paper disks (6 mm), impregnated with known amounts of lysozyme and / or arachidonic acid, were placed on the surface of YNB media plates that were inoculated confluently with a standardized suspension of *E. coli* K-12. The arachidonic acid and lysozyme, which diffuse into the media, caused a zone of inhibition of growth of *E. coli* around the disk.

The protocol was as follows. A sterile cotton swab was placed in *E. coli* K-12 suspension and excess fluid was removed by pressing and rotating the cotton against the inside of the tube above the fluid level. The swab was then streaked in at least three directions over the surface of the YNB media to obtain uniform growth. A final sweep was made around the rim of the plate. The plates were allowed to dry for five minutes. Using sterile forceps, disks containing arachidonic acid and/or lysozyme were applied onto the

plates. The plates were incubated within 15 minutes after application of the disks. Following overnight incubation, the diameter of the zone of inhibition of growth was used as a measure of susceptibility.

The paper disks were impregnated in the following solutions:

A: De-ionized water

B: Arachidonic acid (1 microgram / ml in deionized water)

C: Lysozyme (50 Shugar units / ml in deionized water)

D: Arachidonic acid (1 microgram / ml) + lysozyme (50 Shugar units/ml) in deionized water

The following results were obtained:

Experiment	zone of inhibited growth (mm)
A	0
B	5
C	8
D	23

Comparative Examples 18 and 20 and Examples 19 and to 21

The efficacy of a combination of a mixture of lysozyme and arachidonic acid and phospholipase A_2.

The experimental procedures and basal feed composition was the same as for Examples 1 to 4.

The experiment comprised the following treatments:

(18) basal diet (negative control)

(19) basal diet + lysozyme (50,000 Shugar units/kg of feed) + arachidonic acid (ARA) to a final concentration of 0.001 g/kg of feed.

(20) Basal diet + phospholipase A_2 (500 EYU/kg of feed)

(21) basal diet + lysozyme (50,000 Shugar units/kg of feed) + arachidonic acid (ARA) to a final concentration of 0.001 g/kg of feed + phospholipase A_2 (500 EYU/kg of feed).

The effects of the combination of lysozyme and arachidonic acid and phospholipase A_2 on growth and feed conversion ratio in broilers after 28 days are presented in Table 5.

Table 5

Example	Diet	Feed Intake (g)	Growth (g)	Feed conversion Ratio
18	Basal diet	2173	1319	1.65
19	Basal diet + lysozyme + arachidonic acid	2249	1409	1.59
20	Basal diet + phospholipase	2159	1347	1.60
21	Basal diet + lysozyme + arachidonic acid + phospholipase	2241	1424	1.57

In this trial it was shown that both the combination of lysozyme and arachidonic acid (and phospholipase) significantly improved the performance of the broilers.

CLAIMS

1. An animal feed composition comprising:

(a) a compound that disrupts the peptidoglycan layer of bacteria; and

(b) a compound that disrupts the phospholipid layer of bacteria.

2. An animal feed additive or premix composition comprising:

(a) a compound that disrupts the peptidoglycan layer of bacteria; and

(b) a compound that disrupts the phospholipid layer of bacteria.

3. A composition according to claim 1 or 2 wherein the compound that disrupts the peptidoglycan layer cleaves peptidoglycan or lyses or degrades the peptidoglycan layer.

4. A composition according to any one of the preceding claims wherein the peptidoglycan disrupting compound is a protein, such as an enzyme.

5. A composition according to any preceding claim wherein the peptidoglycan disrupting compound is an endoglycosidase, endoacetylmuramidase, glucosaminidase, an endo- or exo-peptidase, or a carbohydrase.

6. A composition according to any preceding claim wherein the peptidoglycan disrupting compound is an enzyme that is soluble in water and/or thermostable.

7. A composition according to any preceding claim wherein the peptidoglycan disrupting compound hydrolyzes peptidoglycan from the cell wall, for example it cleaves peptidoglycans between muramic acid and acetylglucosamine.

8. A composition according to any preceding claim wherein the peptidoglycan disrupting compound comprises lysozyme, mutanolysin or N-acetylmuramyl-L-alanine amidase.

9. A composition according to any preceding claim wherein the peptidoglycan disrupting compound comprises lysozyme and is at a concentration of 1,000 to 10,000,000, such as from 10,000 to 150,000, more preferably from 25,000 to 100,000 Shugar units per kilogram of animal feed.

10. A composition according to any preceding claim wherein the peptidoglycan disrupting compound comprises lysozyme at a concentration of from 0.04 to 44 milligrams per kg of feed, preferably from 0.4 to 6.7 milligrams per kg of feed, and more preferably from 1.1 to 4.4 milligrams per kg of feed.

11. A composition according to any preceding claim wherein the phospholipid layer disrupting compound comprises a protein, such as an enzyme, or an organic or inorganic compound.

12. A composition according to claim 11 wherein the compound comprises a phospholipase, a polyunsaturated fatty acid (PUFA), or a chelating agent.

13. A composition according to claim 12 wherein the phospholipase is from a mammalian, e.g. human source and/or is PLA_2 and is present at a concentration of from 5 to 5,000, preferably from 10 to 2,500 , and more preferably from 50 to 1,000 Egg Yolk Units per kilogram of animal feed.

14. A composition according to claim 12 wherein the phospholipase is at a concentration from 0.005 to 5 milligrams per kg of feed, preferably from 0.01 to 2.5 milligrams per kg of feed, and more preferably from 0.05 to 1.0 milligrams per kg of feed.

15. A composition according to any of claims 11 to 14 wherein the phospholipid layer disrupting compound is derived from an animal, an animal product, plant, plant product, an algae, algal product, single cell, single cell product or microorganism.

16. A composition according to claim 15 wherein the compound is of microbial origin and/or is recombinant protein.

17. A composition according to claim 12 wherein the PUFA comprises an Ω-3 or Ω-6 C_{18}, C_{20} or C_{22} PUFA.

18. A composition according to claim 12 wherein the PUFA is in the form of free fatty acid, salt, fatty acid ester, phospholipid or mono-, di- or triglyceride.

19. A composition according to claim 12 wherein the PUFA comprises arachidonic acid (ARA).

20. A process for the preparation of a feed composition, suitable for a monogastric or non-ruminant animal, the process comprising adding a compound that disrupts the peptidoglycan layer and a compound that disrupts the phospholipid layer of bacteria to an animal feed, or mixing a feed additive ir orenux composition according to any of claims 2 to 18 with one or more edible feed substance(s) or ingredient(s).

21. An animal feed composition comprising an additive or premix composition according to claim 2 and one or more edible feed substance(s) or ingredient(s).

22. A process for promoting growth and/or feed conversion in a monogastric or non-ruminant animal, the process comprising feeding the animal a compound that disrupts

the peptidoglycan layer and a compound that disrupts the phospholipid layer bacteria or a composition as defined in any of claims 1 or 20.

23. A process according to claim 21 where the animal is monogastric and/or non ruminant such as pig, piglet, poultry, (veal) calf or aquatic animal.

Application No:	GB 0224534.8	**Examiner:**	Dr William Thomson
Claims searched:	1-23	**Date of search:**	22 January 2003

Patents Act 1977 : Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance	
X	1-8, 11,12 and 17-23 at least	WO 00/21381A1	(DSM N.V.) See whole document, in particular page 1, lines 3-6, Examples 4, 5, 9, 10, 14, 15 and claims 1-23
X	1-8, 11, 12 and 17-23 at least	EP 0366869A2	(LYCON AG) See whole document, in particular page 2, lines 3-11 and 42-54, Example 1 and claims 1, 2, 7, 12, 15 and 25
X	1-8, 11 and 12 at least	US 4810508	(DELL' ACQUA ET AL) See whole document, in particular column 1, lines 5-14, Example 10 and claims 1-9

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[V]:

A5B

Worldwide search of patent documents classified in the following areas of the IPC[7]:

A23K; A61K

The following online and other databases have been used in the preparation of this search report:

CAS-ONLINE, EPODOC, JAPIO & WPI

BNSDOCID: <GB_____2379168A_I_>